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Autoregulation of collagenase production by a protein synthesized and secreted by synovial fibroblasts: Cellular mechanism for control of collagen degradation

(phorbol myristate acetate/connective tissue regulation/immunoprecipitation)

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ABSTRACT **Conditioned medium taken from cultures of resting rabbit synovial fibroblasts contained a protein that prevented the synthesis of the neutral proteinase collagenase. Conditioned medium was concentrated 10-fold and placed on cultures of rabbit synovial fibroblasts along with an inducer of collagenase (phorbol myristate acetate or latex particles) and [^{3}H]leucine. Collagenase production was measured by immunoprecipitation of culture medium with monospecific antibody. Gel filtration showed that the inhibitory factor had M_rs of 12,500, 25,000–50,000, and 150,000, suggesting that the protein may exist as aggregates. Activity was destroyed by boiling, by trypsin, and by dithiothreitol. Production of the inhibitory protein was prevented by cycloheximide. Isoelectric focusing purified the protein 100- to 150-fold and revealed pIs in the range of 3.2–3.7. Glycosylation was demonstrated by binding to Con A-Sepharose. Our data indicate that rabbit synovial fibroblasts autoregulate collagenase production and suggest that the low levels of collagenase seen in resting cultures result from an active suppression of collagenase synthesis.**

The interstitial collagens are the body's most abundant structural proteins, and collagen remodeling occurs in a number of normal processes such as uterine resorption and wound healing (1–3) and in diseases such as rheumatoid arthritis and tumor invasion (4, 5) (for reviews, see refs. 6 and 7). The fact that collagen breakdown can be initiated only by the metalloproteinase collagenase (EC 3.4.24.7) makes this enzyme rate-limiting in collagenolysis and assures the importance of cellular mechanisms regulating its production. Although collagenase is produced by a variety of mammalian cell types (6, 7), the mechanisms governing collagenase synthesis and secretion differ. In polymorphonuclear leukocytes (8, 9) and monocytes/macrophages (10–12), collagenase is synthesized and stored within the cell in granules; secretion is regulated by factors controlling granule release rather than by those controlling enzyme synthesis (7). In contrast, fibroblast (e.g., skin and synovial cell) collagenase is not stored. Addition of an inducer stimulates transcription of collagenase mRNA followed by rapid synthesis and secretion of the enzyme (13–17).

An abundant source of enzyme is that produced by fibroblasts derived from the synovial tissue lining the joints (4, 6, 7). Normally, these cells are quiescent, but proliferating synovial tissue in rheumatoid arthritis produces large quantities of collagenase, and the role of this enzyme in the destruction of connective tissues is well documented (4, 6, 7). The fact that these high levels of collagenase can be induced by treating cultures of resting synovial fibroblasts with a variety of stimuli makes these cells an attractive experimental model for studies on mechanisms controlling collagenase synthesis (14, 17–19).

Over the past few years, considerable information has been obtained on the biosynthesis of synovial cell collagenase. Upon addition of a stimulus to cultures of rabbit synovial fibroblasts, increased collagenase mRNA is detected in the cell by 5 hr and collagenase protein appears in the culture medium by 10 hr (14, 17, 19). Studies on the biosynthesis of both synovial and skin collagenase show that the time required for synthesis and secretion is <1 hr (13, 16). Synovial cell collagenase is secreted predominantly as latent procollagenase of M_r 57,000, along with a small amount of a glycosylated species of M_r 61,000 (16, 20). Activation of the latent enzyme occurs through limited proteolysis (7) or through a specific activator protein (7, 21) that is produced by skin, uterine, and synovial fibroblasts.

Despite accumulated knowledge related to mechanisms of collagenase biosynthesis, cellular events and factors that might regulate the synthetic process and hence modulate connective tissue metabolism are poorly understood. Johnson-Wint and Gross have indicated that this regulation may well occur through effector molecules called cytokines (22, 23). They describe a protein produced by corneal epithelial cells that inhibits collagenase production by corneal fibroblasts. In the studies described here, we report that a protein produced by populations of rabbit synovial fibroblasts inhibits collagenase synthesis by these cells.

MATERIALS AND METHODS

Cell Cultures. Monolayer cultures of rabbit synovial fibroblasts were prepared by dissociating synovial tissue excised from the knee joints of freshly killed New Zealand White rabbits (Snelling Rabbitry, Springfield, VT) into a single-cell suspension (24). Cells were plated in Dulbecco's modified Eagle's medium (DME medium; GIBCO) in 20% fetal calf serum (GIBCO) and gentamycin; at confluence, cells were passaged in 10% fetal calf serum.

Preparation of Conditioned Medium and Bioassay for Inhibitory Protein. Confluent monolayers of rabbit synovial fibroblasts in 100-mm-diameter culture dishes were washed three times (5 min per wash) in Hanks' balanced salt solution to remove traces of serum. Seven milliliters of DME medium supplemented with 0.2% lactalbumin hydrolysate were added, and the cultures were incubated for 48–72 hr. Before the conditioned medium was harvested, sample portions from each dish were tested for collagenolytic activity (18, 25). Medium from cultures showing no enzyme activity in an 18-hr assay was harvested and stored at −20°C. Medium was concentrated by precipitation with 80% saturated ammonium

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Abbreviation: PMA, phorbol myristate acetate.

sulfate. The precipitate was suspended in 0.05 M ammonium bicarbonate and dialyzed exhaustively vs. this solution using dialysis tubing with a cutoff of M_r 3500. Dialyzed material was lyophilized.

To test the medium for biologic activity, we measured its ability to inhibit collagenase synthesis by immunoprecipitating [^{3}H]collagenase protein from culture medium. Lyophilized samples were reconstituted 10 times concentrated in leucine-free medium (to 1.5–2.0 mg of protein per ml), sterilized by filtration, and placed on confluent cultures of synovial fibroblast in 35-mm-diameter culture dishes along with an inducer of collagenase [phorbol myristate acetate (PMA) at 10 nM; Consolidated Midland, Brewster, NY] and [^{3}H]leucine (6.7 Ci/mmol; 20 μCi/ml, New England Nuclear, Boston; 1 Ci = 37 GBq). After 24–30 hr at 37°C, cultures were terminated by removing the medium and washing the cells twice with cold 5% CCl_3COOH. The CCl_3COOH-precipitable proteins were solubilized in 1 ml of 0.2 M NaOH for determination of protein content by the method of Lowry *et al.* (26).

Preparation of Antisera and Immunoprecipitation, NaDodSO$_4$/PAGE, and Autoradiography. Antisera monospecific for rabbit synovial cell collagenase and activator were prepared in sheep as described by Vater *et al.* (21, 27). Briefly, crude culture medium containing rabbit synovial fibroblast collagenase was treated with sheep anti-rabbit synovial fibroblast collagenase antiserum, a gift of C. Vater and E. D. Harris, Jr., in a double-immunodiffusion assay in agar. The resulting immunoprecipitin bands were excised from the agar, emulsified in complete Freund's adjuvant, and injected subcutaneously into a North Country Cheviot sheep (21, 27). Fig. 1 shows that the resultant antiserum is monospecific for rabbit synovial cell collagenase by its single line of identity with the antiserum known to be monospecific for this antigen (21, 27) (Fig. 1 *Left*), by its ability to form a single band against crude culture medium containing rabbit synovial collagenase in a crossed immunoelectrophoresis assay (Fig. 1 *Center*), and by its ability to neutralize the activity of rabbit synovial fibroblast collagenase (Fig. 1 *Right*). Antiserum to rabbit activator was prepared in an identical manner and was shown to be monospecific by the same criteria (21).

Synthesis of immunoreactive collagenase was detected in culture medium as described previously (16).

Gel Filtration. Concentrated conditioned medium was applied to Ultrogel AcA 44 or AcA 54 equilibrated in phosphate-buffered saline containing either 0.15 M or 1 M NaCl. Fractions (2 ml) were collected at 12 ml/hr, and absorbance at 280 nm was determined. Fractions were pooled in groups of three, dialyzed vs. 0.05 M ammonium bicarbonate, lyophilized, resuspended in 2 ml of leucine-free medium with [^{3}H]leucine, and assayed for their ability to inhibit collagenase synthesis.

Isoelectric Focusing and Con A-Sepharose Chromatography. Concentrated culture medium was dialyzed vs. 0.05 M ammonium bicarbonate and lyophilized. Lyophilized material, containing ≈20 mg of protein, was suspended in a small volume of water and subjected to isoelectric focusing (21).

Appropriate fractions isolated from the isoelectric focusing column pH 2.5–4 were pooled, and subjected to Con A-Sepharose chromatography as described in ref. 21.

Materials. Trypsin, soybean trypsin, inhibitor, dithiothreitol, and cycloheximide were purchased from Sigma. Latex particles were from Difco.

RESULTS

Inhibition of Collagenase Synthesis by Conditioned Medium. Fig. 2 shows that when 10-times-concentrated conditioned medium was placed on cultures of fibroblasts, collagenase production was inhibited. In Fig. 2*A*, lanes 1 and 2 represent the negative control of immunoprecipitable collagenase synthesized by cells treated with nonconditioned medium. Lanes 3 and 4 represent the positive control of collagenase synthesized by cells treated with nonconditioned medium in the presence of the collagenase inducer PMA. Lanes 5 and 6 show collagenase produced by cells treated with ×10 conditioned medium and PMA. Compared with the positive control, there is less collagenase synthesized by cultures treated with the conditioned medium. Fig. 2*B* shows that the pattern of proteins synthesized and secreted by all groups of cells is similar, demonstrating that the decrease in collagenase production is not the result of a generalized toxic effect on protein synthesis. In support of this idea, the protein content of the control and experimental cultures is indistinguishable (1049 ± 117 μg).

The suppressive effect of conditioned medium was quantified in three experiments (Table 1). In all experiments, treatment with nonconditioned medium and PMA elevated collagenase well above control cultures, and treatment with conditioned medium antagonized the effect of PMA by reducing collagenase levels nearly to those of the control cultures. To show that the inhibition was not due to an effect of the condi-

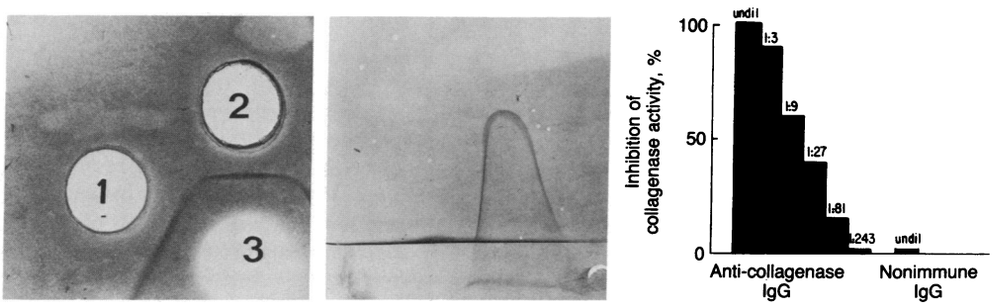

FIG. 1. Characterization of monospecific antibody to rabbit synovial fibroblast collagenase. Sheep anti-rabbit synovial fibroblast collagenase antiserum was prepared and tested for monospecificity as described in *Materials and Methods* and in refs. 21 and 27. Antiserum was tested for monospecificity by double immunodiffusion assay (*Left*), by crossed immunoelectrophoresis (*Center*), and by neutralization of collagenase activity (*Right*). (*Left*) Wells: 1, reference standard of sheep antirabbit synovial fibroblast collagenase antiserum (21, 27); 2, our preparation of sheep anti-rabbit synovial fibroblast collagenase antiserum; 3, 20-times-concentrated crude culture medium from PMA-stimulated cells. (*Center*) Thirty microliters of crude (×20) concentrated culture medium from PMA-stimulated rabbit synovial fibroblasts was electrophoresed in 1% agarose at pH 8.6. Separated antigens were then electrophoresed for the second dimension into agarose containing our antiserum to rabbit synovial fibroblast collagenase. (*Right*) Culture medium from PMA-stimulated rabbit synovial fibroblasts was activated with trypsin (21, 27). Samples of activated culture medium were mixed with various dilutions of our preparation of sheep anti-rabbit synovial fibroblast collagenase IgG (undiluted = 2.3 mg of protein per ml) or with undiluted nonimmune IgG (2.7 mg of protein per ml) for 1 hr at 37°C and then overnight at 4°C. Reaction mixtures were centrifuged, and the supernatants were assayed for collagenase activity in a 7-hr assay with fibrils of radiolabeled collagen substrate (21, 27).

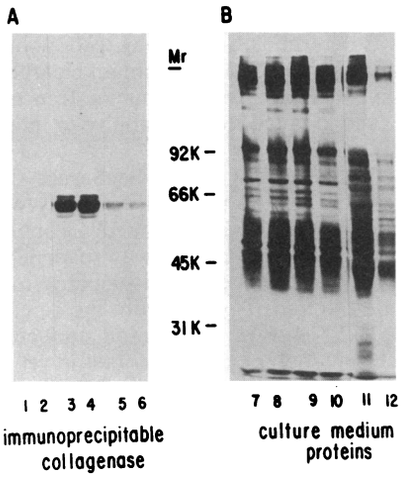

FIG. 2. Inhibition of collagenase synthesis by conditioned medium. Duplicate cultures of rabbit synovial fibroblasts were treated with 10-times-concentrated conditioned medium, prepared as described, PMA (10 nM) and [^{3}H]leucine. Duplicates of control cultures received nonconditioned medium without PMA (negative control) or with it (positive control) and [^{3}H]leucine. After 30 hr at 37°C, synthesis of [^{3}H]collagenase was measured by immunoprecipitation (*A*), and the pattern of culture medium proteins was visualized (*B*) by gel electrophoresis and autoradiography. Lanes: 1, 2, 7, and 8, nonconditioned medium; 3, 4, 9, and 10, nonconditioned medium with PMA; 5, 6, 11, and 12, ×10 conditioned medium with PMA.

tioned medium on the PMA, we tested the ability of conditioned medium to inhibit induction by latex beads (100 μg/ml) (28). In this experiment, treatment with PMA or latex induced 555 ± 87 or 286 ± 13 cpm of immunoprecipitable collagenase, respectively. Concomitant treatment with PMA and ×10 conditioned medium or with latex and ×10 conditioned medium reduced the cpm of immunoprecipitable collagenase to 122 ± 16 for PMA and to 47 ± 4 for latex.

To show that resting cultures were actively synthesizing and secreting the inhibitory factor, we treated cell cultures with cycloheximide at 5 μg/ml for 48 hr. This medium was harvested, processed as described earlier, and placed on cells for its ability to inhibit collagenase synthesis in the presence of PMA. Table 2 shows that conditioned medium from cycloheximide-treated cultures failed to inhibit induction of collagenase synthesis. The cycloheximide treatment was not toxic to cells producing the conditioned medium: levels of lactate dehydrogenase in the culture medium were not elevated, and the protein content of the cycloheximide-treated cultures was 75% of control cultures.

Determination of Molecular Weight and Characterization. The molecular weight of the inhibitory factor was determined by fractionating conditioned medium on Ultrogel AcA

Table 1. Quantitation of the inhibition of collagenase synthesis by conditioned medium (CM)

Treatment of cells	[^{3}H]Collagenase, cpm		
	Exp. I	Exp. II	Exp. III
×1 non-CM	375 ± 19	321 ± 38	566 ± 130
×1 non-CM with PMA	3152 ± 93	920 ± 51	934 ± 50
×10 CM with PMA	766 ± 134	609 ± 44	297 ± 94

In three experiments, duplicate cultures were treated with 10-times-concentrated conditioned medium, prepared as described, along with PMA (10 nM) and [^{3}H]leucine. Control cultures received nonconditioned medium (non-CM), with or without PMA, and [^{3}H]leucine. Cultures were incubated and assayed as described in Fig. 1, and the amount of immunoprecipitable [^{3}H]collagenase was quantified. Data are means ± SD.

Table 2. Effect of cycloheximide on the synthesis of inhibitory protein

Treatment of cells	[^{3}H]Collagenase, cpm
×1 non-CM	150
×1 non-CM with PMA	485
×10 CM with PMA	103
×10 CM (cycloheximide-treated cells) with PMA	627

Ten-times-concentrated conditioned medium was prepared from parallel cultures that were or were not treated for 48 hr with cycloheximide (5 μg/ml). The medium was assayed for its ability to inhibit the synthesis of [^{3}H]collagenase. [^{3}H]Collagenase was immunoprecipitated from culture medium and quantified.

44 in physiologic saline. Fractions were pooled in groups of three, dialyzed, lyophilized, and reconstituted in leucine-free medium with [^{3}H]leucine and PMA and were assayed for their ability to inhibit induction of collagenase synthesis. Fig. 3 *Left* shows that fractions containing protein with M_r 150,000, 25,000, and 12,500 contained inhibitory activity. A second experiment with Ultrogel AcA 54 and 1 M salt (Fig. 3 *Right*) confirmed the presence of inhibitory activity at several areas across the column. Similar to the data shown in Fig. 3 *Left* activity was seen at M_r 150,000 and 12,500, but in this experiment inhibition occurred predominantly at M_r 50,000 rather than at M_r 25,000, suggesting the possible existence of aggregated protein.

We further characterized this inhibitory protein by subjecting it to a variety of experimental conditions. Inhibitory activity of the ×10 conditioned medium was destroyed by boiling for 10 min, by trypsin (100 μg/ml for 60 min at RT, followed by addition of soybean trypsin inhibitor) or by 10 mM dithiothreitol (for 30 min). Addition of trypsin and soybean trypsin inhibitor to cultures in the absence of PMA failed to induce collagenase, thus indicating that the presence of large amounts of these proteins could not stimulate collagenase synthesis (data not shown).

Isoelectric Focusing and Con A-Sepharose Chromatography. Initial experiments used an isoelectric focusing column and ampholytes with the pH range of 6–8, with extreme pH gradients seen at each end of the column. Fractions from the column were pooled in groups of five and were assayed for their ability to inhibit collagenase production. Fig. 4 *Upper* shows that fractions containing protein with pIs in the range of 3–5 inhibited induction of collagenase.

Since activator protein is important in the regulation of enzymatically active collagenase (7, 21) and since both collagenase and activator are found in medium from induced culture (21), we tested whether the material preventing collagenase synthesis could also diminish production of activator. Fig. 4 *Lower* shows (*i*) that the same fractions capable of inhibiting collagenase production also prevent production of activator and (*ii*) that the inhibitory activity was destroyed by boiling. Fractions taken from other areas of the column did not affect activator synthesis (data not shown).

Isoelectric focusing with ampholytes in the range of pH 2.5–4 was used to further fractionate the inhibitory protein (Fig. 5 *Upper*). In three experiments, major areas of inhibitory activity focused in the range of pI 3.2–3.7 and were purified at least 100- to 150-fold.

The variation in pI could indicate heterogeneity in glycosylation. To demonstrate glycosylation, we measured the ability of the inhibitory fractions to bind to Con A-Sepharose (Fig. 5 *Lower*). The figure shows that cells treated with non-inhibitory fractions produced large amounts of collagenase and that most of the activity in the inhibitory fraction bound to Con A. However, some inhibitory material did not bind, and this may reflect additional heterogeneity in the kinds of carbohydrate residues present.

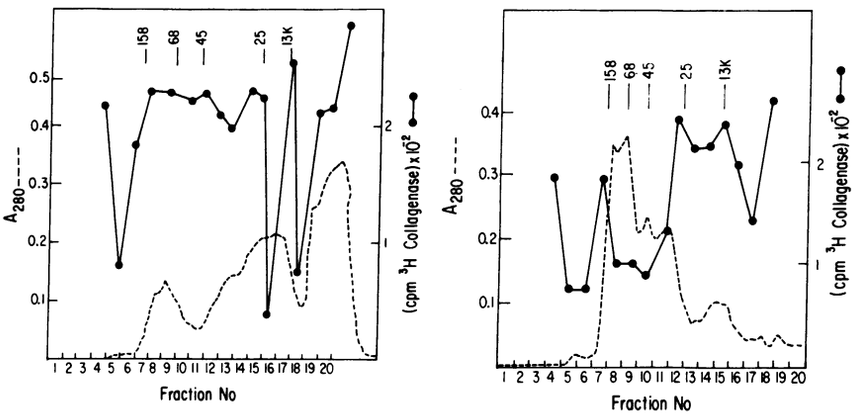

FIG. 3. Gel filtration of protein inhibiting collagenase synthesis. Conditioned medium was concentrated, dialyzed, lyophilized, and resuspended in phosphate-buffered saline containing NaCl at 0.15 M (*Left*) or 1 M (*Right*) and passed through Ultrogel AcA 44 (*Left*) or AcA 54 (*Right*). Fractions were pooled in groups of three, dialyzed, lyophilized, and reconstituted in leucine-free medium with [^{3}H]leucine and assayed for their ability to inhibit the induction of [^{3}H]collagenase in the presence of PMA (10 nM). [^{3}H]-Collagenase was immunoprecipitated from culture medium and quantified.

DISCUSSION

In this paper, we describe a protein that is synthesized and secreted by rabbit synovial cells and that inhibits the production of both collagenase and its activator by these cells. It is an autoregulatory protein in the sense that it is a protein produced by mesenchymal cells and that acts on these same cells. In the absence of cloned populations of synovial fibroblasts, it is impossible to determine whether or not the inhibitory protein is, in fact, produced by one subset of synovial fibroblasts and acts to prevent collagenase synthesis by another subset. The protein appears not to be stored, since protein synthesis is required for its activity. It is destroyed

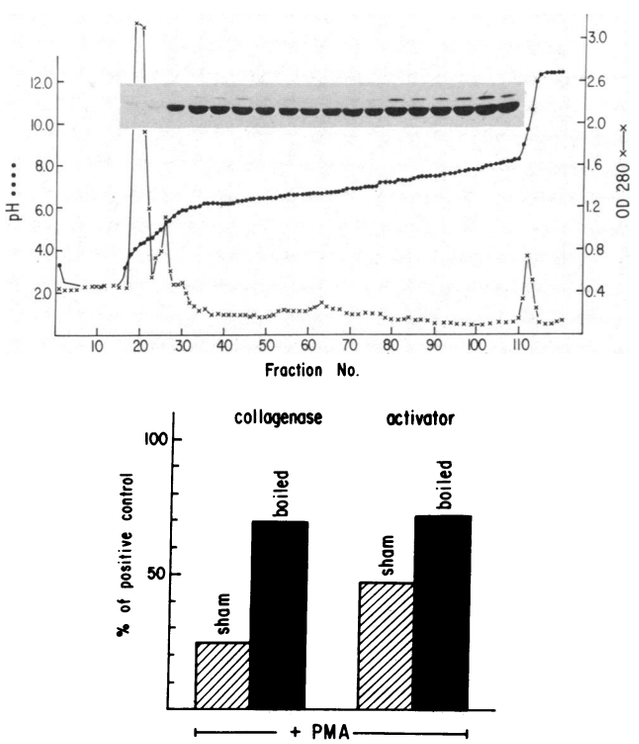

FIG. 4. Isoelectric focusing of protein inhibiting synthesis of collagenase and activator. (*Upper*) Inhibition of collagenase synthesis. Conditioned medium (containing ≈20 mg of protein) that had been dialyzed and concentrated was applied to an isoelectric focusing column with ampholytes in the pH range of 6–8. Fractions (1 ml) were collected, pooled in groups of five, and assayed by immunoprecipitation for their ability to inhibit synthesis of [^{3}H]collagenase in the presence of PMA (10 nM). (*Lower*) Inhibition of collagenase and activator synthesis and effect of boiling on inhibitory activity. The two fractions containing inhibitory activity were pooled. Each pool was divided in half: one-half was boiled for 10 min, and the other half was sham-treated. After dialysis vs. leucine-free medium, the fractions were placed on virgin fibroblasts, and medium from these cultures was assayed by immunoprecipitation for the amount of [^{3}H]collagenase and [^{3}H]activator synthesized.

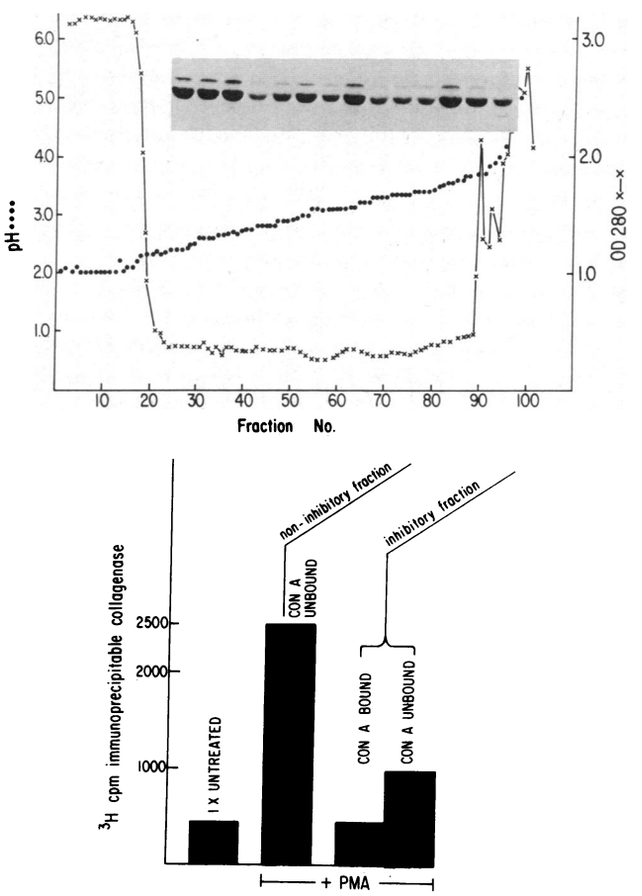

FIG. 5. Isoelectric focusing of protein inhibiting collagenase synthesis. (*Upper*) Inhibition of collagenase synthesis. Conditioned medium (containing ≈20 mg of protein) that had been dialyzed and concentrated was applied to an isoelectric focusing column with ampholytes in the pH range of 2.5–4. Fractions (1 ml) were collected, pooled in groups of five, and assayed by immunoprecipitation for their ability to inhibit the synthesis of [^{3}H]collagenase in the presence of PMA (10 nM). (*Lower*) Binding of inhibitory protein to Con A-Sepharose. Three fractions containing inhibitory activity were pooled, as were three adjacent noninhibitory fractions. Each pool was passed through a Con A-Sepharose column equilibrated with 50 mM Tris, pH 8.0/0.15 M NaCl, and bound material was eluted with 0.5 M α-methylmannoside. After dialysis and lyophilization, bound and unbound fractions, each containing 150 μg of protein, were tested for their ability to inhibit the synthesis of [^{3}H]collagenase in the presence of PMA (10 nM).

by boiling, by trypsin, and by dithiothreitol. It has apparent M_rs of 12,500, 25,000–50,000, and 150,000 and may exist as aggregates. Partial purification was achieved by isoelectric focusing and Con A-Sepharose chromatography. The relatively low pI of this protein (3.2–3.7) suggests that it may be heavily glycosylated, and this was confirmed by affinity chromatography with Con A-Sepharose. The broad range over which this inhibitory protein focused indicates heterogeneity in glycosylation, as is known to occur in a number of proteins, such as immune interferon (29), synovial cell collagenase (16), and interleukin 2 (30).

In our system, the inhibitory protein acts as an autoregulatory protein, actively maintaining a constitutive level of collagenase synthesis by resting cultures. This concept of an active suppression of collagenase production contrasts with the supposition that the absence of collagenase in resting cultures correlated with the absence of a stimulus (7, 18) and was therefore a "passive" phenomenon. To date, most studies on the regulation of collagenolysis have been concerned with compounds that modulate collagenase activity (6, 7) rather than synthesis. These include proteases such as plasmin (31), trypsin (32, 33), kallikrein (34), and, more recently, activator (21) that activate the latent enzyme. Conversely, there are numerous and nearly ubiquitous specific and nonspecific protease inhibitors of active collagenase (7). These include α_2-macroglobulin (35, 36), ovostatin (37, 38), and a specific inhibitor of metalloproteinases that is produced by skin (39), lung, corneal, and tendon fibroblasts as well as by osteoblasts and smooth muscle cells (40). It is probably identical to the β_1 anti-collagenase inhibitor found in serum (7, 40), and its abundance suggests that it may be an important mechanism for regulating the activity of metalloproteinases in connective tissues throughout the body (7, 40).

A protein acting at a level other than the proteolysis-based mechanism and capable of regulating the actual synthesis of collagenase has important implications for connective tissue biology and pathology. The previous description of an inhibitory protein capable of controlling production of corneal collagenase (22, 23) and our description here of a similar factor operative in the control of the synthesis of synovial cell collagenase suggest that, similar to the inhibitor of metalloproteinase activity, this type of regulatory protein may be fairly widespread throughout the body. This suggestion receives support from the fact that collagenase produced by synovial and skin fibroblasts share a variety of biochemical and biological characteristics. Both exist in doublet form (13–17, 41, 42) and are rapidly synthesized and secreted (13, 16), and, most importantly, synthesis of both can be regulated by the same growth factors and hormones (7, 18, 43–45).

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